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Paul E. Waggoner
A. E. Dimond

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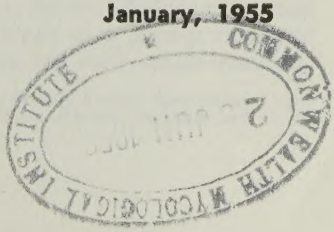

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ASSAYING EFFECT OF GROWTH REGULATORS UPON PLANT TUMORS

Paul E. Waggoner and A. E. Dimond

An examination of methods for detecting effects of growth regulators upon plant tumors is the purpose of this bulletin.[1] Crown gall, caused by *Agrobacterium tumefaciens* on a large number of dicotyledonous plants, is a neoplasm widely studied because of its analogy to animal tumors. Many of these studies have been concerned with the effects of growth regulators, such as hormones or ionizing radiation, upon gall growth. Hence, in the case of this tumor, data are available for comparing the relative efficiencies of different methods of assaying growth regulators. This comparison will permit the cost of an experiment to be calculated from the cost per experimental unit, the convenience of manipulation, and the number of replications necessary to discriminate between treatment means. The results are essentially applicable to studies of other tumors, *e.g.*, insect galls and virus tumors.

Tissue cultures and galls on intact plants and on storage organs have been used for assays. Tissue cultures *in vitro* have several advantages: "1) Simple, callus-like tissue may be grown vegetatively *in vitro* for unlimited periods free from variation arising from pollination. 2) Since the nutrients consist only of known chemicals, the basal metabolism can be studied with relatively simple and controlled procedures. 3) Changes in both kind and character of cells may be induced under controlled conditions. 4) The tissue may be exposed a long time to a wide range in concentrations of materials. 5) It is possible to determine how far the tissue develops by itself or is dependent on other parts of the plant." (Hildebrandt and Riker, 1947). Galls formed on the stems of a plant growing in the greenhouse lack certain of these advantages. However, the galls form rapidly and can be studied with the crudest of equipment. In addition, the method presents an opportunity for continuous, quantitative measurements of neoplastic cells growing in the otherwise normal host. Finally, galls formed on storage organs, *e.g.*, carrot root slices, combine many of the advantages of tissue culture with rapidity of gall formation and low cost per unit.

IN VITRO CULTURE OF TISSUE

Data showing the quantity of information available from experiments with tissues cultured *in vitro* have been published by deRopp (1947). He grew bacteria-free gall and normal sunflower and periwinkle tissue for 28 days on the medium used by White and Braun (1942). The media contained 0, .01 and 10 ppm indole acetic acid. Thus, the variabilities of normal and gall tissue from two hosts exposed to three levels of a growth regulator can be compared. When the standard deviations for each tissue-treatment

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combination are plotted against the corresponding means (Figure 1), it is seen that a single proportionality fits the data from sunflower and *Vinca*, normal and gall tissue. That is, $s = .4\ \bar{x}$ accounts for 99.6 per cent of the variability in s where s and $\bar{x}$ are the sample standard deviation and mean. In contrast to this, the relation between sample variance and mean, $s^2 = .08\ \bar{x}$, accounts for only 74 per cent of the variability in s^2. Bartlett (1947) has discussed the case of standard deviation proportional to mean. When this occurs, it is advantageous to transform the weights into logarithms. We may then be fairly confident that the analysis of variance is applicable and that treatment effects will be more nearly additive. If some treatments suppress galls altogether, log (weight + 1) can be used. If deRopp's 12 x 20 observations were pooled in a single analysis of variance without transformation, the expected standard error of a mean would be $(.4)\ \bar{x}/\sqrt{20}$ or 20 mg. with treatment means from 42-670. The expected standard error of a mean log (weight) would be $.17/\sqrt{20}$ or .04 with treatment means from 1.62-2.82. Thus, for several types of tissue culture on media containing different levels of a growth regulator the estimated error variance was constant when weights were transformed into logarithms. With this variance, about 38 unpaired replicates would be required to obtain a "t" significant at .05 for a 20 per cent difference between two means.

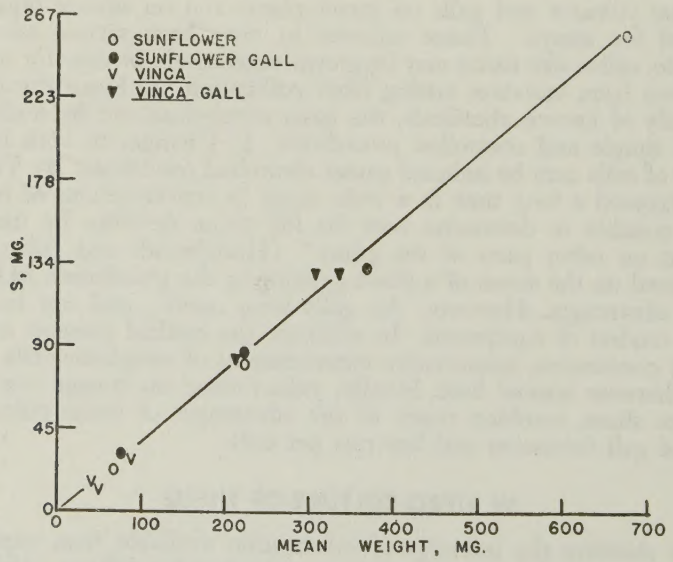

Figure 1. Sample means and standard deviations for *in vitro* tissue culture weights. DeRopp (1947)

The data of Hildebrandt, Riker and Duggar (1945) and Hildebrandt and Riker (1947) can be compared with deRopp's. The former (1945) have published the weights of six replicates of tobacco tissue grown for 42 days on a basic medium (White, 1942) with sucrose from 0 to 16 per cent.

If the variances for each treatment are estimated and plotted against the means, a proportionality is seen that accounts for 69 per cent of the variability of s^2. However, the proportionality between s and $\bar{x}$, $s = .15\ \bar{x}$, accounts for somewhat more, 79 per cent. Therefore, weights should be transformed to log (weight), a result consistent with that from deRopp's data. However, the proportionality constant for tobacco tissue data, .15, is smaller than for deRopp's tissue data, .40. When the tobacco tissue weights are transformed into logs, the error mean square is changed from 2700 mg.[2] with means from 110-450 mg. to .0053 with means from 1.04-1.65. The variance ratio or F for treatment means is increased from 35 to 58.

Although the transformation suggested for deRopp's and the tobacco data is the same, the error variance for a given mean is larger for the sunflower and *Vinca* than for the tobacco tissue. Whereas, we saw above that 38 unpaired replicates of the tissue employed by deRopp would have been required to obtain a significant "t" for a 20 per cent difference between means, only about seven would be required for the tobacco tissue.

Whether or not the ratio between mean and standard deviation will sometimes vary between laboratories, times, and tissues can be decided by examining the data of Hildebrandt and Riker (1945). They grew sunflower gall tissue and the same isolate of tobacco as they used earlier on a basic medium to which 0-100 ppm of 2,4-dichlorophenoxyacetic acid was added. The error mean squares, 15,000 for sunflower and 800 mg.[2] for tobacco, can be calculated from the published means and F values. The ratios of standard deviation to mean are .37 for sunflower and .24 for tobacco. Consequently, the expected variances for transformed data are .026 and .011 for sunflower and tobacco, respectively. When .030, the error mean square for logs of sunflower gall tissue weights from deRopp's data, is compared to .026, that from Hildebrandt and Riker (1947), it is seen that there is no significant difference. On the other hand, the error mean square for logs of tobacco tissue weights are not the same from one time to another, even in the same laboratory, varying from .0053 to .0110.

In summary, experiments involving tissue cultures have shown that it is advantageous to transform weights to log (weight). Tobacco tissues provided error mean squares of .010-.005 when logs were used. Studies from two laboratories using different media, on isolates of sunflower and *Vinca* galls or normal tissue, and with different growth regulators and growth periods show surprising uniformity. Data transformed to logarithms in all experiments have error mean squares not significantly different from .03. This result suggests that an experiment can be designed to demonstrate the significance of any given percentage change in tissue weight. For example, about 38 unpaired replicates would be required to obtain a significant "t" for a 20 per cent difference between means.

Insufficient data are published to estimate increases in efficiency due to restrictions on randomization. Some general statements can be made. An increase in incubation temperature from 20 to 24° C. causes up to 100 per cent increase in tissue weight (Hildebrandt, Riker, and Duggar, 1945). Therefore, if a temperature gradient exists in the incubator, its effects should

be removed from the error mean square by means of a randomized block or Latin square design. The variance values for sunflower gall and tobacco tissue published by Hildebrandt and Riker (1947) are smaller for experiments run at a single time than for ones run at several times. This suggests that the effect of "times" should be removed from the error sum of squares. When different temperatures or times are used as replicates, a hypothesis of broader application would be tested if one used as an error term "treatment x replicates" rather than the "within treatment and replicate" mean square.

CULTURE OF GALLS ON PLANT STEMS

Data showing the quantity of information available from experiments with galls grown on plant stems have been published by Waggoner and Dimond (1952). They exposed tomato, *Nicotiana glutinosa,* and kalanchoe plants to 2 or 30 Kr (1000 roentgens) of gamma radiation immediately after or five days before inoculation. The stems were inoculated at three points and published data are the antilogs of the mean logs of the volumes of the three galls formed 32-34 days after inoculation. Thus, we can estimate the variability of gall growth on three hosts at two doses of a growth regulator, ionizing radiation. When the standard deviations are plotted against the means, (Figure 2), a single relation fits the data for all three hosts. This relation, s= .52 $\bar{x}$, accounts for 87 per cent of the variability in s.

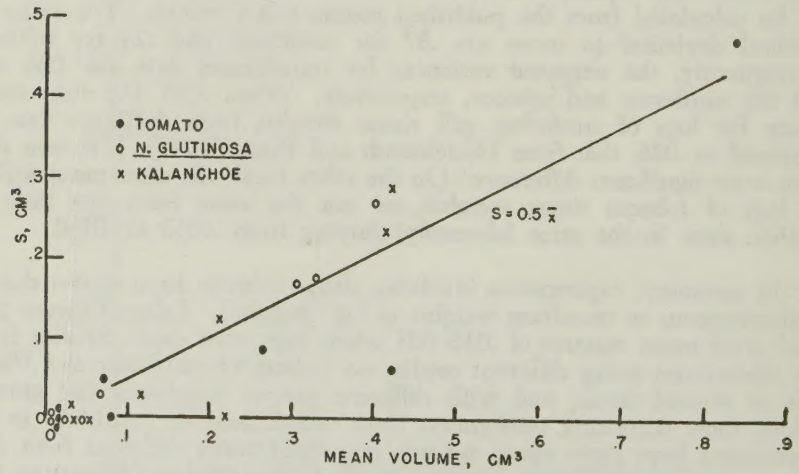

Figure 2. Sample means and standard deviations for crown gall volumes. Waggoner and Dimond (1952)

Therefore, as in the case of *in vitro* tissue weights, treatment effects will be more nearly additive if gall measurements are converted into logs. If the preceding observations are transformed into logs, the error mean square is about .05. This is significantly greater than .03, the error mean square for sunflower or *Vinca* tissue.

The error variance for log (tomato gall volumes) does not appear to be as consistent from one experiment to another as in the case of tissue culture. Sample variances range from .02 when young plants are treated to as high as .23 when older plants are treated. In order to obtain a "t" significant at .05 for a 20 per cent difference between two means of unpaired replicates, 25 replicates would be required with the younger plants and 290 with the older ones. These should be compared to 38, the number of replicates of tissue cultures required.

Unpublished data can be used to evaluate restrictions on randomization. In the following experiments some treatments resulted in no galls. Therefore, one was added to the measurements before transformation into logarithms. Five-week-old, four-leaf Bonny Best tomato plants were subjected to 9 Kr of soft X-rays at 5 r/sec. No part, the roots, the third internode, the growing point, or the entire plant were exposed. The plants were inoculated by a needle puncture in the third internode and then arranged in two 5 x 5 Latin squares in such a manner that each treatment appeared once in each column of 5 and twice in each row of 10. The bench contained sand and a constant-level water table. Nutrients were added three times a week. Plant heights were measured after six and eighteen days. Gall volumes were estimated after eighteen days by measuring gall and stem diameter. The analysis of variance of the log (volumes) is given in Table 1. Two missing log (volumes) in different treatments, rows, and columns were estimated (Yates, 1933).

Table 1. Analysis of Variance of Log (Volumes) of Galls on Tomato Stems 18 Days After Inoculation

Source of variation	Degrees of freedom	Mean square	Variance ratio
Columns	9	.63	2.7*
Rows	4	.26	1.1
Irradiation	4	1.56	6.7**
Error	30	.23	
Total	47		

There were significant differences among the column totals but not among the row totals. The efficiencies of a randomized block with columns as blocks and complete randomization relative to the Latin square design were 99 and 73 per cent, respectively. That is, only about three-quarters as many plants would be required to achieve a particular accuracy if a randomized block design were used rather than a complete randomization. The Latin square was no more efficient than a randomized block design.

Sometimes the efficiency of an experiment can be increased by using concomitant information. For example, the host plant that grows more rapidly during the period of gall growth would likely produce a larger gall.

$$\frac{\text{host volume at day 18}}{\text{host volume at day 6}} = K \text{ (gall volume at day 18).}$$

Taking the cube of the host height as proportional to host volume,

$$h^3_{18} / h^3_{6} = K'/v$$

where h_i is host height at day i and v is gall volume. This becomes

$$\log (h_{18}/h_6) = K'' + \tfrac{1}{3} \log v.$$

A significant regression of $\log (h_{18}/h_6)$ on log v was found within the five irradiation treatments described above. However, the error term was decreased only 12 per cent. This does not appear to be a highly successful means of increasing efficiency.

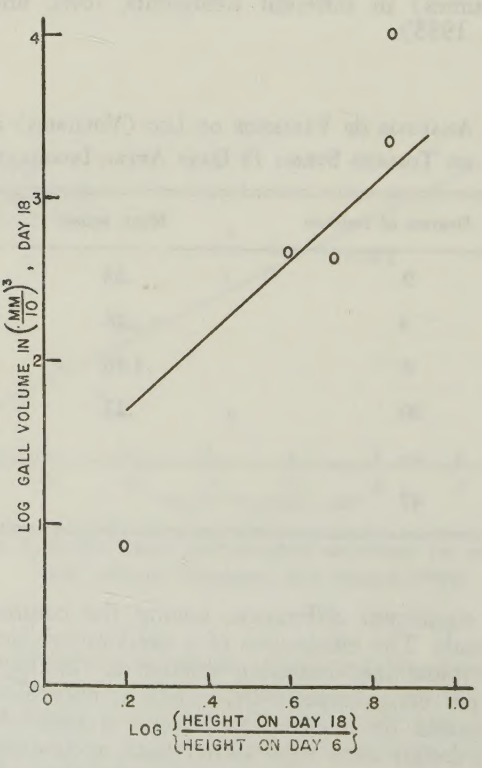

Figure 3. Change in host and gall volumes for tomato plants.

The adjustment for host growth affected the significance of the difference between mean gall volumes for the irradiation treatments. As Figure 3 shows, 83 per cent of the variation in gall volume produced by irradiation is accounted for by the variation in host volume produced. The remaining variation is not significantly greater than the error term. Hence, irradiation must suppress galls by stunting all parts of the host, as suggested by Waggoner and Dimond (1952).

Further unpublished data is available to show the effects of confounding, independent variable, and observation time upon quantity of information. Eighty three-week-old Bonny Best tomato plants were arranged in five replicates of 16 plants each. Each of the 16 combinations of four inoculation dates and four dates of treatment with the diethanolamine salt of maleic hydrazide (MH) were applied to a plant in each replicate. The main effects of inoculation date were confounded, i.e., whole plots of four plants were inoculated on a single date. The plants were inoculated by a needle puncture in the internode above the cotyledon, at 3, 5 or 7 weeks of age or not at all. The plants were sprayed with 5.36×10^{-3}M MH at 3, 5 or 7 weeks of age or not at all. Gall diameters were measured weekly from 4 to 10 weeks. Wet and dry weights of galls on the 60 inoculated plants were determined at 10 weeks.

Irrespective of the variable (log of volume, wet weight, or dry weight) the results at week 10 were essentially the same. The mean log (gall volumes) are given in Table 2. (One missing value was estimated by method of Anderson, 1946).

TABLE 2. MEAN LOG GALL VOLUME IN $\frac{mm^3}{10}$ WHEN BONNY BEST TOMATO PLANTS WERE 10 WEEKS OLD

Inoculation	Spray of 5.36 x 10-3 M MH at			
	3 weeks	5 weeks	7 weeks	None
3 weeks	2.90	5.09	5.63	6.74
5	2.69	2.78	4.73	5.18
7	3.11	4.28	3.77	4.79
Mean	2.90	4.04	4.70	5.66

Standard errors: Between means for MH treatment: week 3 and others, .62; others, .27. Between means for MH treatment at a single inoculation time: treatment week 3 and others at inoculation week 7, 1.11; between others, .45.

MH reduced gall volume whether applied before or after inoculation. As Greulach (1951) has shown, the effect of MH is greatest on three-week-old plants. This is also true of the gall tissue on the plant.

The analyses of variance for the three variables assuming homogeneity of variance are given in Table 3.

TABLE 3. ANALYSES OF VARIANCE OF GALL MEASUREMENTS ON BONNY BEST TOMATOES 10 WEEKS OLD

Source of variation	Df	Mean squares or (variance ratios)		
		Log (volume)	Log (wet weights)	Log (dry weights)
Replicate	4	(2.66)	(<1)	(<1)
Inoculation (I)	2	(16.50)**	(8.85)**	(10.66)**
Error a	8	.0580	.3576	.2066
MH	3	(38.70)**	(53.80)**	(49.50)**
I x MH	6	(4.85)**	(2.44)*	(2.30)*
Error b	35	.0582	.2047	.1378
Total	58			

From the analyses we conclude that the restrictions on randomization were of limited value. Further, although the error mean squares for volumes were smaller than for dry weights which in turn were less than for wet weights, the differences between means decreased in the same way. Therefore, the variance ratios were not materially changed by change in variable.

The error mean squares fluctuated with different times of treatment, inoculation, and observation. They were at a maximum with plants more than five weeks old when galls were beginning to appear and part of them were recorded as O. By treating and inoculating plants three weeks old and measuring galls at five weeks, error variances are equal to or less than those obtained with *in vitro* tissue cultures.

If older plants are used, treatment differences are more difficult to discriminate for two reasons. First, the differences are smaller. Second, the error terms are larger. Therefore, if older plants are used one might well fail to discover the effect of a regulator such as MH. This may explain the failure of Klein and Klein (1952) to observe gall suppression by MH.

The discussion of experiments with galls growing on plants in the greenhouse can be briefly summarized. Data should be transformed into logarithms. Host or method of measuring gall size do not appear to be important. Error mean squares are at a minimum when galls are apparent

on all (or none) of the plants receiving a single treatment and when young plants are used. Growth periods as short as one week can be used. Restrictions on randomization have increased efficiency as much as one-fourth. Error variances run from .02 for three-to-four-week-old plants, to .23 for older plants as compared to a relatively consistent .03 for *in vitro* tissue culture. Thus, fewer replicates of young plants or more replicates of old plants would be required than *in vitro* cultures to obtain a significant "t" for a given difference between means.

CULTURE OF GALLS ON STORAGE ORGANS

We can now turn to storage organs which combine some of the advantages of *in vitro* tissue culture with simplicity and economy. No nutrient media need be provided, the cost of the hosts for an experiment is usually less than a dollar, and growth periods of two weeks can be used. Both carrot roots and Jerusalem artichoke tubers have been used (deRopp, 1950). Quantitative data suitable for statistical analysis is available from the authors' unpublished work. Differences in ability to produce galls exist between roots (deRopp, 1950), but this source of variation is easily removed from the error variance by a restriction upon randomization.

A demonstration that a randomized block design where roots are the blocks is more efficient than complete randomization is given by the following experiment. A carrot root was surface-sterilized, cut transversely into eight slices .5 cm. thick, and a slice placed apical-end down on 3 per cent water agar in each of eight petri plates. Klein (1954) has confirmed the advisability of using sections of the same thickness with the same end up. The apical end was set down in our experiments to permit movement of regulators to the region of gall growth. Similar slices from 14 other roots were also placed in plates. The surfaces of all slices were inoculated at the same time with an aqueous suspension of *A. tumefaciens*. One slice from each root was transferred at a given time from the water agar to another plate where the agar solution contained 2.67×10^{-3} M MH. The times of transfer were 7, 5, 1, or 0 days before and 1, 5 or 7 days after inoculation. A check remained on water agar. Fourteen days after inoculation no galls had formed on the slices transferred to MH at or before inoculation. The gall tissue was removed from the other carrots and weighed. Only one of the 15 slices transferred one day after inoculation produced a gall. The mean log (gall fresh weight in mg.) was .270 for transfer at 5 days, .530 at 7 days, and 1.293 for no transfer. The analysis of variance of the log (gall weights) for these last three treatments shows that transfer to MH 5 or 7 days after inoculation suppresses the galls significantly (Table 4). The means for earlier transfer are less than the means for 5 and 7 days by an amount greater than the LSMD calculated from Table 4. The randomized block design doubled efficiency in this experiment because the root to root variation was large.

Table 4. Analysis of Variance of Log (Gall Weights) 14 Days After Inoculation of Carrot Slices

Source of variation	Df	Mean square	Variance ratio
Root	14	1.43	3.86**
MH	2	4.24	11.50**
Error	28	.37	
Total	44		

Latin square designs have been used in which each treatment is applied to a section from each root and to a section from each height on the upper portion of the roots. The ability to form galls does not appear to differ from one height to another in this case. However, sections from the most basal part of the root do have decreased ability to produce galls (Klein, 1954). Therefore, these sections should be discarded or, if used, a Latin square design should be employed.

Error variance has been found to be largest when galls have formed on some, but not all, of the slices receiving a single treatment. In this case there is a discontinuity in the scale of measurement between zero and the smallest size that can be removed from the carrot and weighed.

In a number of experiments the gall sizes have been ranked as well as weighed. The ranks have then been analyzed by the method of Wilcoxon (undated) or have been transformed into the expected values from a normal distribution as tabulated by Fisher and Yates (1948). The ranking methods have had essentially the same ability as the weighing method to discriminate between treatment means.

The characteristics of experiments using carrot roots can be summarized briefly. Growth periods of two weeks can be used. Error variances are at a minimum when measurable galls are present on all (or none) of the slices receiving a single treatment. When a randomized block design with roots as blocks is used, efficiency is increased. Only sections from the apical half of the root should be used or a Latin square design employed. Sections of uniform thickness should be set with the same end up.

Mean squares for the interaction of treatments and roots when log (weights) are measured have varied from .12 to .40. These compare to the error mean square given above for tissue culture, .03, and for young tomatoes, .02. More carrot slices than young tomatoes or tissue cultures must be used to demonstrate the significance of a given percentage difference. However, they may be no more variable than older tomatoes.

SUMMARY

An examination has been made of the relative efficiencies of assaying growth regulator effects upon tumor tissue by means of tissue culture, galls on intact hosts, and galls on storage organs. The standard deviation was found to be proportional to the mean. Hence, a logarithmic transformation is advantageous. *In vitro* cultures of sunflower and *Vinca* gall and normal tissue exposed to various growth regulators in different laboratories resulted in an error mean square of .03. When galls were grown on intact hosts or on storage organs, the error mean square was smallest when galls were apparent on all (or none) of the plants receiving a single treatment. In the case of galls grown on intact hosts, the host or method of measuring gall size did not appear to be important. Inoculation and treatment of three-week-old tomato plants resulted in a smaller error mean square, .02, than when older plants were used. Error mean squares as large at .23 appeared when five-week-old plants were used. Arranging plants in randomized blocks increased efficiency up to 25 per cent. Galls grown on carrot slices provided error mean squares between .12 and .40 when individual roots were arranged as replicates. Because of the low cost of the carrot slices, it may be more economical to use many carrot slices than a few tissue cultures or tomato plants.

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